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Spectral sensitivity of guppy visual pigments reconstituted *in vitro* to resolve association of opsins with cone cell types

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ABSTRACT

The guppy (*Poecilia reticulata*) shows remarkable variation of photoreceptor cells in the retina, especially those sensitive to middle-to-long wavelengths of light. Microspectrophotometry (MSP) has revealed varying "green", "green-yellow" and "yellow" cone cells among guppies in Trinidad and Venezuela (Cumana). In the guppy genome, there are four "long-wave" opsin loci (*LWS-1*, *-2*, *-3* and *-4*). Two *LWS-1* alleles have potentially differing spectral sensitivity (*LWS-1*/180$_{Ser}$ and *LWS-1*/180$_{Ala}$). In addition, two "middle-wave" loci (*RH2-1* and *-2*), two "short-wave" loci (*SWS2-A* and *-B*), and a single "ultraviolet" locus (*SWS1*) as well as a single "rhodopsin" locus (*RH1*) are present. However, the absorption spectra of these photopigments have not been measured directly and the association of cell types with these opsins remains speculative. In the present study, we reconstituted these opsin photopigments *in vitro*. The wavelengths of maximal absorbance (λ_{max}) were 571 nm (*LWS-1*/180$_{Ser}$), 562 nm (*LWS-1*/180$_{Ala}$), 519 nm (*LWS-3*), 516 nm (*LWS-2*), 516 nm (*RH2-1*), 476 nm (*RH2-2*), 438 nm (*SWS2-A*), 408 nm (*SWS2-B*), 353 nm (*SWS1*) and 503 nm (*RH1*). The λ_{max} of *LWS-3* is much shorter than the value expected (560 nm) from the "five-sites" rule. The two *LWS-1* alleles could explain difference of the reported MSP λ_{max} values for the yellow cone class between Trinidad and Cumana guppies. Absence of the short-wave-shifted *LWS-3* and the green-yellow cone in the green swordtail supports the hypothesis that this cell class of the guppy co-expresses the *LWS-1* and *LWS-3*. These results reveal the basis of variability in the guppy visual system and provide insight into the behavior and ecology of these tropical fishes.

1. Introduction

The guppy (*Poecilia reticulata*) shows remarkable polymorphism in male body coloration (Breden, 2006). Recent studies suggest that ambient light variation between microhabitats can influence female preference of different male signal combinations, thus explaining the evolution and persistence of color variation in males (Cole & Endler, 2015; Seehausen, 2015). However, the degree of variability in the visual system and its effect on female preference for male color pattern remains poorly understood.

A microspectrophotometry (MSP) study of the Trinidad guppy revealed cone photoreceptor cells in the retina with λ_{max} values of 389 nm ("ultraviolet"), 408 nm ("violet") and 464 nm ("blue") as well as rod cells of 501 nm (Archer & Lythgoe, 1990). Another recent study of the Venezuelan (Cumana) guppy reported a similar result [359 nm (ultraviolet), 406 nm (violet), 465 (blue) and 503 nm (rod)] (Watson et al., 2011). It has long been known through MSP studies that, in comparison to the short-wavelength cone classes and the rod cells, the cone classes in the middle-to-long wavelength range are highly variable in spectral sensitivity among individual guppies (Archer, Endler, Lythgoe, & Partridge,

Abbreviations: λ_{max}, wavelengths of maximal absorbance; M/LWS, middle-to-long-wavelength-sensitive; MSP, microspectrophotometry; PCR, polymerase chain reaction; RH1, rhodopsin or rod opsin; RH2, rhodopsin-like middle-wave-sensitive; RT, reverse transcription; SWS1, short-wavelength-sensitive type 1; SWS2, short-wavelength-sensitive type 2.

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1987; Archer & Lythgoe, 1990; Watson et al., 2011). When pooling λ_{max} values recorded from multiple cells in multiple individuals, λ_{max} distribution centers around three peaks, 533, 548 and 572 nm, in a study of the Trinidad guppy (Archer & Lythgoe, 1990). A study of the Cumana guppy reported a consistent result in which λ_{max} distributions are centered at 525 nm ("green"), 541 nm ("green-yellow") and 560 nm ("yellow"), slightly shorter than in the former study (Watson et al., 2011).

The 548-centered distribution of λ_{max} values found in the Trinidad guppy is remarkably broader than the 533- and 572-centered distributions, implying that the cells centered at the 548-nm peak represent a cell group that expresses both 533- and 572-nm opsin genes in the same cell, in varying ratios (Archer & Lythgoe, 1990). In the study of the Cumana guppy, the λ_{max} distribution of the 541-nm class cones ranges over 10 nm (minimum of 536 nm to maximum of 546 nm) and is broader than other classes (<5 nm), though authors note their small sample size (Watson et al., 2011). This spectral variation is not likely attributed to differences in chromophore (A1 vs A2) use among cells because the MSP absorption spectra of the guppy cones are well fitted by an A1 chromophore template (Archer & Lythgoe, 1990; Archer et al., 1987). A similar result is also reported for congeneric molly species (*Poecilia mexicana*, *P. latipinna* and *P. formosa*), for which long-wavelength cone classes generally show relatively high variance (Korner, Schlupp, Plath, & Loew, 2006). It is noted, however, that the green-yellow cone class is absent in the green swordtail (*Xiphophorus helleri*), a species closely related to the guppy, while the green and the yellow cones are observed (Watson, Lubieniecki, Loew, Davidson, & Breden, 2010).

In the guppy genome, there are four cone opsin genes belonging to the M/LWS (middle-to-long-wavelength-sensitive) class (Hoffmann et al., 2007; Ward et al., 2008; Watson et al., 2011; Weadick & Chang, 2007), *LWS-1*, *LWS-2*, *LWS-3* and *LWS-4*: the nomenclature follows conventions established by Sandkam et al. (2013) and Tezuka et al. (2014). The *LWS-1*, *LWS-2* and *LWS-3* genes are physically linked in this order, but orientation of the *LWS-3* is opposite to the others (Watson et al., 2010, 2011). The *LWS-4* lacks introns other than intron 1 and is located in a different linkage group (Ward et al., 2008; Watson et al., 2010, 2011). Two cone opsin genes belong to the SWS2 (short-wavelength-sensitive type 2) class, *SWS2-A* and *SWS2-B*, and are located in tandem in upstream of *LWS-1* (Watson et al., 2010, 2011). Additionally, guppies have two cone opsin genes belonging to the RH2 (rhodopsin-like middle-wave-sensitive) class, *RH2-1* and *RH2-2*, along with a single *SWS1* (short-wavelength-sensitive type 1) cone opsin gene, and a single *RH1* (rhodopsin or rod opsin) gene (Hoffmann et al., 2007). Thus, there are more opsin types than known cell types reported for cones.

Quantitative polymerase chain reaction (qPCR) of retinal RNA has revealed that in adult guppy retinae the expression level of *SWS2-A*, *LWS-2* and *LWS-4* is relatively low (Laver & Taylor, 2011; Sandkam, Young, & Breden, 2015). Study of *in situ* hybridization of adult guppies has revealed that *LWS-4*-positive cells are found confined to the peripheral dorsal–temporal retina (Rennison, Owens, Allison, & Taylor, 2011). In contrast, *SWS2-A* cells, as well as *SWS-1*, *SWS2-B* and *RH2-2* cells, are observed throughout the retina. *LWS-2* cells, as well as *LWS-1* and *LWS-3* cells, are distributed broadly in the central to dorsal area of the retina. *RH2-1* cells are observed throughout the retina but with higher density in ventral area (Rennison et al., 2011).

Among vertebrates, λ_{max} values of M/LWS opsins can generally be inferred from the amino acid composition at five sites (the "five-sites" rule): Ser or Ala at 180 (designated as $180_{Ser/Ala}$), $197_{His/Tyr}$, $277_{Tyr/Phe}$, $285_{Thr/Ala}$ and $308_{Ala/Ser}$ (the residue number follows that in human M/LWS opsins) (Yokoyama & Radlwimmer, 1998; Yokoyama, Yang, & Starmer, 2008). Two *LWS-1* alleles of the guppy differing at site 180 are reported: one (*LWS-1*/180_{Ala}) with Ala, His, Tyr, Thr and Ala at 180, 197, 277, 285 and 308, respectively (designated as Ala/His/Tyr/Thr/Ala) (Tezuka et al., 2014; Watson et al., 2011), and the other (*LWS-1*/180_{Ser}) with Ser/His/Tyr/Thr/Ala (Tezuka et al., 2014). The five-site composition of *LWS-2* is Pro/His/Phe/Ala/Ala and those of *LWS-3* and *LWS-4* are both Ser/His/Tyr/Thr/Ala (Watson et al., 2011). The Ser-to-Pro mutation ($_{Ser}180_{Pro}$) is reported to shift the λ_{max} by 19 nm toward a shorter wavelength for the lamprey LWS photopigment (Davies, Collin, & Hunt, 2009). By following the five-sites rule (Yokoyama et al., 2008) and assuming the putative $_{Ser}180_{Pro}$ effect, we can infer λ_{max} values of the guppy M/LWS opsins (the second column of Table 1).

The λ_{max} values of other guppy opsins can be inferred from the values of the closest relative for which opsins have been measured *in vitro*, the medaka (*Oryzias latipes*). The guppy and the medaka both belong to the Superorder Cyprinodontea and belong to the Orders Cyprinodontiformes and Beloniformes, respectively (Nelson, 2006). The measured λ_{max} values of the medaka opsins are: 356 nm (*SWS1*), 405 nm (*SWS2-B*), 438 nm (*SWS2-A*), 452 nm (*RH2-A*), 502 nm (*RH1*), 492 nm (*RH2-B*), 516 nm (*RH2-C*) and 561–562 nm (*LWS-A* and *LWS-B*) (Matsumoto, Fukamachi, Mitani, & Kawamura, 2006). The guppy *RH2-2* is orthologous to the medaka *RH2-A*, and the guppy *RH2-1* is orthologous to the medaka *RH2-B* and *RH2-C* clade (Owens, Windsor, Mui, & Taylor, 2009).

Taking into consideration the inferred λ_{max} values of the opsins and their expression pattern, a possible association of the opsins to the MSP cell types is given in the third column of Table 1. One to one association of the cell with the opsin is reasonably inferable only for: 1) the violet cone with *SWS2-B*, 2) the ultraviolet cone with *SWS1*, and 3) the rod with *RH1*. In other cases, matching of the λ_{max} values between the inferred opsins and the observed cells is poor, or multiple opsins are inferred to have similar λ_{max} values. Although the assignment of *LWS-1*/180_{Ala} to the green-yellow cone is also suggested by previous studies based on the inferred λ_{max} and its high expression level (Laver & Taylor, 2011; Watson et al., 2011), this is opposed to the hypothesis that the green-yellow cones express two opsin types (Archer & Lythgoe, 1990). Archer and Lythgoe (1990) showed that the absorption curves of the yellow and green cones fit well to one-opsin templates while that of the green-yellow cone did not. They do, however, fit well to a mixture model. Assignment of an *RH2* opsin to the green cone has also been suggested by previous studies (Hofmann & Carleton, 2009; Laver & Taylor, 2011; Watson et al., 2010). If the co-expression of the green-cone opsins and the yellow-cone opsins within the green-yellow cone type is true, it is unclear how an RH2 opsin gene (*RH2-1*) and an M/LWS gene are co-regulated, because the transcriptional regulation over two opsin classes (*RH2* and *LWS*) would not be more likely than regulation within each opsin class (*RH2* or *LWS*) (Tam et al., 2011; Tsujimura, Chinen, & Kawamura, 2007; Tsujimura, Hosoya, & Kawamura, 2010; Tsujimura, Masuda, Ashino, & Kawamura, 2015). Additionally, it is yet to be resolved why the green swordtail lacks the green-yellow cones, despite possessing *RH2-1* and *LWS-2* (*P180*) in the genome (Watson et al., 2010). To contribute to an improved understanding of the visual ecology of tropical fishes, we conducted *in vitro* reconstitution of the guppy visual opsins and measured their absorption spectra.

2. Materials and methods

2.1. Animal protocols

The University of Tokyo Animal Care and Use Committee and Tohoku University approved all animal protocols in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Table 1
Predicted and measured λ_{max} values of guppy visual opsins and associated MSP cell classes inferred from the opsin λ_{max}.

Opsin (Accession number)	Predicted λ_{max} (nm)	Possible cell class[a] (λ_{max}: nm)	Measured λ_{max} (nm)	Possible cell class[b] (λ_{max}: nm)
LWS-1/180$_{Ser}$ (AB748985)	560[c]	Yellow (560[g]–572[h])	571 ± 0.6	"Trinidad (T):" Yellow (572[h])
LWS-1/180$_{Ala}$ (AB748984)	555[d]	Green-Yellow (541[g]–548[h])	562 ± 0.6	"Cumana (C)" Yellow (560[g])
LWS-2 (LC127183)	515[e]	Green (525[g]–533[h]) central/dorsal retina [i]	516 ± 6.9	Green (525[g]–533[h]) central/dorsal retina [i]
LWS-3 (LC127184)	560[c]	Yellow (560[g]–572[h])	519 ± 2	"T" Green-Yellow (548[h]) with *LWS-1*/180$_{Ser}$ "C" Green-Yellow (541[g]) with *LWS-1*/180$_{Ala}$
LWS-4 (LC127185)	560[c]	Yellow (560[g]–572[h])	ND	–
RH2-1 (LC127186)	492–516[f]	Green (525[g]–533[h]) ventral retina [i]	516 ± 1	Green (525[g]–533[h]) ventral retina [i]
RH2-2 (LC127187)	452[f]	Blue (464[h]–465[g])	476 ± 2	Blue (464[h]–465[g])
SWS2-A (LC127188)	438[f]	?	438 ± 0.7	?
SWS2-B (LC127189)	405[f]	Violet (406[g]–408[h])	408 ± 1.3	Violet (406[g]–408[h])
SWS1 (LC127190)	356[f]	UV (359[g]–389[h])	353 ± 2	UV (359[g]–389[h])
RH1 (LC127191)	502[f]	Rod (501[h]–503[g])	503 ± 1	Rod (501[h]–503[g])

[a] Inferred based on the predicted λ_{max} values of the opsins.
[b] Inferred based on the measured λ_{max} values of the opsins.
[c] Inferred from the five-sites rule (Ser/His/Tyr/Thr/Ala) (Yokoyama et al., 2008).
[d] Inferred from the five-sites rule (Ala/His/Tyr/Thr/Ala) (Yokoyama et al., 2008).
[e] Inferred from the five-sites rule (534 nm for Ser/His/Phe/Ala/Ala: Yokoyama, Yang, & Starmer, 2008) and the putative $_{Ser}180_{Pro}$ effect of −19 nm.
[f] λ_{max} values of orthologous medaka opsins (Matsumoto et al., 2006).
[g] λ_{max} value taken from Watson et al. (2011).
[h] λ_{max} value taken from Archer and Lythgoe (1990).
[i] Based on the *in situ* hybridization study by Rennison et al. (2011).

2.2. cDNA isolation

We isolated total RNA from eyes of an adult guppy individual (*Poecilia reticulata*) obtained from an introduced population in the Gabusoka River (Okinawa prefecture, Japan). The molecular phylogeny and genetic divergence of this population is well examined (Shoji, Yokoyama, & Kawata, 2007). We isolated cDNA containing the entire coding sequence for all the guppy opsin genes by reverse transcription (RT) PCR, using the forward and reverse primers complementary to the region immediately upstream of the initiation codon and immediately downstream of the stop codon, respectively (Table 2). The nucleotide sequences of the primers were designed according to published sequences of the Cumana guppy opsin genes (*SWS2-A*, *SWS2-B*, *LWS-1*, *LWS-2* and *LWS-3*: GenBank accession number HM540108; *LWS-4*: HM540107) (Watson et al., 2011) and to our unpublished screening of the λ-phage-vectored genome libraries of guppies from the Gabusoka River and Shimoda (Shizuoka prefecture, Japan). These libraries were probed with medaka visual opsin sequences (Matsumoto et al., 2006). We cloned the PCR products into pBluescript II (SK-) plasmid vector (Stratagene, La Jolla, CA) and were subjected to nucleotide sequencing of both strands by an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems Japan, Tokyo). The coding nucleotide sequences were confirmed by duplicate RT-PCRs.

The entire coding sequences were recloned into the pMT5 expression vector (Khorana, Knox, Nasi, Swanson, & Thompson, 1988) following an established procedure (Kawamura & Yokoyama, 1998). We then modified the *LWS-1* clone using QuickChange Site-Directed Mutagenesis Kit (Stratagene, Tokyo, Japan) so as to create two amino acid sequence types that were identical to the *LWS-1*/180$_{Ser}$ (55$_{Val}$/171$_{Gly}$/180$_{Ser}$/247$_{His}$) and *LWS-1*/180$_{Ala}$ (55$_{Val}$/171$_{Ala}$/180$_{Ala}$/247$_{Arg}$) reported in Tezuka et al. (2014). Allelic variation is reported for the other *LWS* opsins (*LWS-2*, *LWS-3* and *LWS-4*), *SWS2-B* and *SWS1* by Tezuka et al. (2014). Our sequences corresponded to 72$_{Gly}$/224$_{Val}$/241$_{Ala}$/302$_{Val}$ (*LWS-2*), 131$_{Phe}$/216$_{Val}$/275$_{Ile}$ (*LWS-3*), 26$_{Ser}$/115$_{Phe}$/175$_{Phe}$ (*LWS-4*),

Table 2
RT-PCR primers for guppy opsin cDNAs.

Primer	Sequence (5′ → 3′)
LWS-1 5FL FOR	CTGAGAAACCTTCTTTCCAG
LWS-1 3FL REV	ACAGGTCCAAAACAGAGAAC
LWS-2 5FL FOR	GGGCAGGGGGGAAGTGTA
LWS-2 3FL REV	GACAAAACTTGCAGTCATGC
LWS-3 5FL FOR	AACCTTCTTTCCAGATCAGG
LWS-3 3FL REV	AAAACAGAGAACATGAAGGT
LWS-4 5FL FOR	AGCTCAGATCGTCTTTCCAA
LWS-4 3FL REV	TTAAAAAAACAACAGAGGAT
RH2-1 5FL FOR	TCAAATCACAGCCTGAGAAG
RH2-1 3FL REV	AAGATATGTAGCTGTAATCA
RH2-2 5FL FOR	CCCACTCACAAGGAAACTAG
RH2-2 3FL REV	CATGATTAAGACACATGGTCC
SWS2A 5FL FOR	AGTTTCACGGTTCAGAAAGGAAGCGAA
SWS2A 3FL REV	GCATTTGCAATTAATAGAGGATTACGGTCAGA
SWS2B 5FL FOR	TCCAGTCTAGTTGAAAGTTTTATTCAAAAA
SWS2B 3FL REV	AGATCTTAAGAGGGTCCAACTTT
SWS1 5FL FOR	CGGAGGAACTCAGGGTAAAG
SWS1 3FL REV	TCTGAGTTTTGCTCCAAACA
RH1 5FL FOR	AGCAACCACAAGCCGCAACC
RH1 3FL REV	GCTTGTGCCTGTTGCTCCAT

$52_{Phe}/62_{Val}/223_{Thr}/272_{Met}/281_{Val}$ (*SWS2-B*) and 171_{Tyr} (*SWS1*) at these amino acid sites. The DDBJ/EMBL/GenBank accession numbers given to the sequences used for reconstitution of photopigments in this study are listed in Table 1.

2.3. Reconstitution of opsin photopigments

For the reconstitution of non-*LWS* class opsin photopigments, we followed a conventional procedure (Kawamura & Yokoyama, 1998; Matsumoto et al., 2006). In brief, the pMT5 clones were transfected into cultured COS-1 cells (RIKEN Cell Bank, Tsukuba, Japan). We incubated cells with 5 μM 11-*cis* retinal (Storm Eye Institute, Medical University of South Carolina, Charleston), and solubilized them with 1% dodecyl maltoside. We purified the reconstituted photopigments using immobilized 1D4 antibody (Molday & MacKenzie, 1983) (University of British Columbia, Vancouver). For *LWS* opsins, we shortened the incubation duration after transfection from 55–63 h to 50–51 h, in accordance with a study on mice reporting that a significant amount of mislocalized M opsin was degraded 55 h after transfection (Zhang, Zhang, Baehr, & Fu, 2011). The absorption spectra of the photopigments were recorded across 250–750 nm in 0.5 nm intervals using the U3010 dual beam spectrometer (Hitachi Ltd., Tokyo) at 20 °C. Measurements were made a minimum of five times in dark and at least five more times after 3 min of light exposure from a 60-watt room lamp (in the case of *RH1*, *RH2*s and *LWS*s) with Kodak Wratten Gelatin Filter No.3, which cuts off wavelengths shorter than 440 nm, or exposure to 350-nm UV light (in the case of *SWS1* and *SWS2*s). Recorded spectra were analyzed and the difference spectra between before and after irradiation were calculated with UV Solution Software (Hitachi Ltd., Tokyo).

We also reconstituted the *LWS-2* and *LWS-3* photopigments following the method described by Imai, Terakita, and Shichida (2000) with some modifications. Briefly, opsins were expressed in HEK293T cells and reconstituted with 11-*cis* retinal after harvesting the cells. Pigments were extracted with buffer *E* (50 mM HEPES, 0.75% CHAPS, 1 mg/ml phosphatidylcholine, 140 mM NaC1) under the red light (>670 nm) at 4 °C. A spectrophotometer (Model UV-2450, Shimadzu Co., Ltd, Kyoto, Japan) was used to measure the UV–visible spectra. For these measurements, samples containing 10 mM hydroxylamine were irradiated for 5 min with light from a 1-kW tungsten halogen lamp (Rikagaku Seiki) that had been passed through a glass cutoff filter (omitting light $\leqslant$520 nm). Difference spectra between before and after irradiations were calculated with Igor Pro software (Wavemetrics, Portland, OR).

3. Results

For eight opsins (*LWS-1*/180_{Ser}, *LWS-1*/180_{Ala}, *RH2-1*, *RH2-2*, *SWS2-A*, *SWS2-B*, *SWS1* and *RH1*) we directly estimated the λ_{max} from absorption spectra measured in dark conditions (the fourth column of Table 1, Figs. 1 and 2). We validated this as follows: in the dark spectra, a peak at 280 nm appears due to absorbance of proteins. When we exposed the reconstituted pigments to light, a new absorption peak appeared at ~380 nm, represented as a negative peak in the dark–light difference spectra (insets of Figs. 1 and 2). This indicates that light isomerized 11-*cis* retinal in the pigments and the resultant all-*trans* retinal was released, demonstrating that the reconstituted pigments were photo-reactive. We verified the ultraviolet sensitivity of *SWS1* pigment by acid denaturation as in Chinen, Hamaoka, Yamada, and Kawamura (2003).

The difference spectrum is useful in the case of a low absorption peak in the dark spectrum. For two opsins (*LWS-2* and *LWS-3*) we estimated the λ_{max} by using the difference spectra (the fourth column of Table 1, Fig. 1). For *LWS-4* opsin, we failed to obtain recognizable peaks even from difference spectrum and despite repeated trials.

4. Discussion

We reconstituted the visual opsins of the guppy *in vitro* and measured their absorption spectra. Difficulties in functional reconstitution of opsins have been reported for heterologous gene expression assays, possibly due to malfunction of cellular processes such as post-translational modifications, trafficking and localization (Chinen, Matsumoto, & Kawamura, 2005; Reeves, Callewaert, Contreras, & Khorana, 2002; Reeves, Kim, & Khorana, 2002; Zhang et al., 2011). In this study, reconstitution of the *LWS* opsins was particularly challenging. By testing several cell lines and culture conditions, the absorption peak of *LWS-1*/180_{Ser} and *LWS-1*/180_{Ala} became prominent in the dark spectra and that of *LWS-2* and *LWS-3* became detectable in the difference spectra. Reconstitution of *LWS-4* remained unsuccessful and may require co-introduction of assisting genes such as those encoding receptor-transporting protein family as seen in the case of olfactory receptors (Zhuang & Matsunami, 2008).

Regarding *SWS1*, *SWS2*, *RH2* and *RH1* genes, the observed λ_{max} values were mostly concordant with those of the medaka orthologs (see the second and the fourth columns of Table 1). For *LWS-2*, the observed λ_{max} value (516 nm) was concordant with prediction by the five-sites rule and consideration of $_{Ser}180_{Pro}$ effect (515 nm). On the contrary, the observed λ_{max} value of *LWS-3* (519 nm) deviated largely from prediction by the five-sites rule (560 nm). Deviation was also notable in *LWS-1*/180_{Ser} (571 nm observation vs 560 nm prediction) and *LWS-1*/180_{Ala} (561 nm vs 555 nm).

Amino acid replacements responsible for these deviations from the five-sites rule should be clarified in future studies by site-directed mutagenesis as in Matsumoto et al. (2014). Here we report that the guppy *LWS-3* contains an amino acid replacement at a highly conserved site, $_{Tyr}178_{Phe}$ (the site number corresponds to that in bovine rhodopsin). This site falls within a track of amino-acid sequence, 174Gly-Trp-Ser-Arg-Tyr178, in the second intradiscal/extracellular loop that is highly conserved in vertebrate visual opsins, and is essential to opsin structure (Doi, Molday, & Khorana, 1990). Thus, this site would be an ideal candidate for the mutagenesis experiment. Interestingly, *LWS-3* of the green swordtail (*Xiphophorus helleri*) (*S180-2*) has Tyr at this site and differs from *LWS-1* (*S180-1*) at only one amino acid site (Val in *S180-1* and Ile in *S180-2* at their 62nd residue located in the first transmembrane domain) (GenBank GQ999832). The λ_{max} value of the green swordtail *LWS-3* is hence predicted be identical to that of its *LWS-1*.

Previously, on the basis of inferred λ_{max} values, *LWS-3* and/or *LWS-4* (λ_{max} both inferred to be at ~560 nm) were suspected to be expressed in yellow cones, *LWS-1*/180_{Ala} (~555 nm) in green-yellow cones and *LWS-2* (~515 nm) or *RH2-1* (492–516 nm) in green cones (Hofmann & Carleton, 2009; Laver & Taylor, 2011; Watson et al., 2010, 2011). This assignment appears consistent with an observation that the green swordtail is reported to lack the green-yellow cones and to have 180_{Ser} fixed in *LWS-1* (i.e. *LWS-1*/180_{Ala} is not found) (Watson et al., 2010, 2011). However, the assignment of a single gene (*LWS-1*/180_{Ala}) to the green-yellow cones appears not compatible with the broad distribution of λ_{max} of this cone class (Archer & Lythgoe, 1990; Korner et al., 2006; Watson et al., 2011) and with the poor match between the absorption curve of the cone class and the single-opsin template (Archer & Lythgoe, 1990). Archer and Lythgoe (1990) proposed the co-expression model of the yellow and the green cone opsins in the green-yellow cones to account for the large variance.

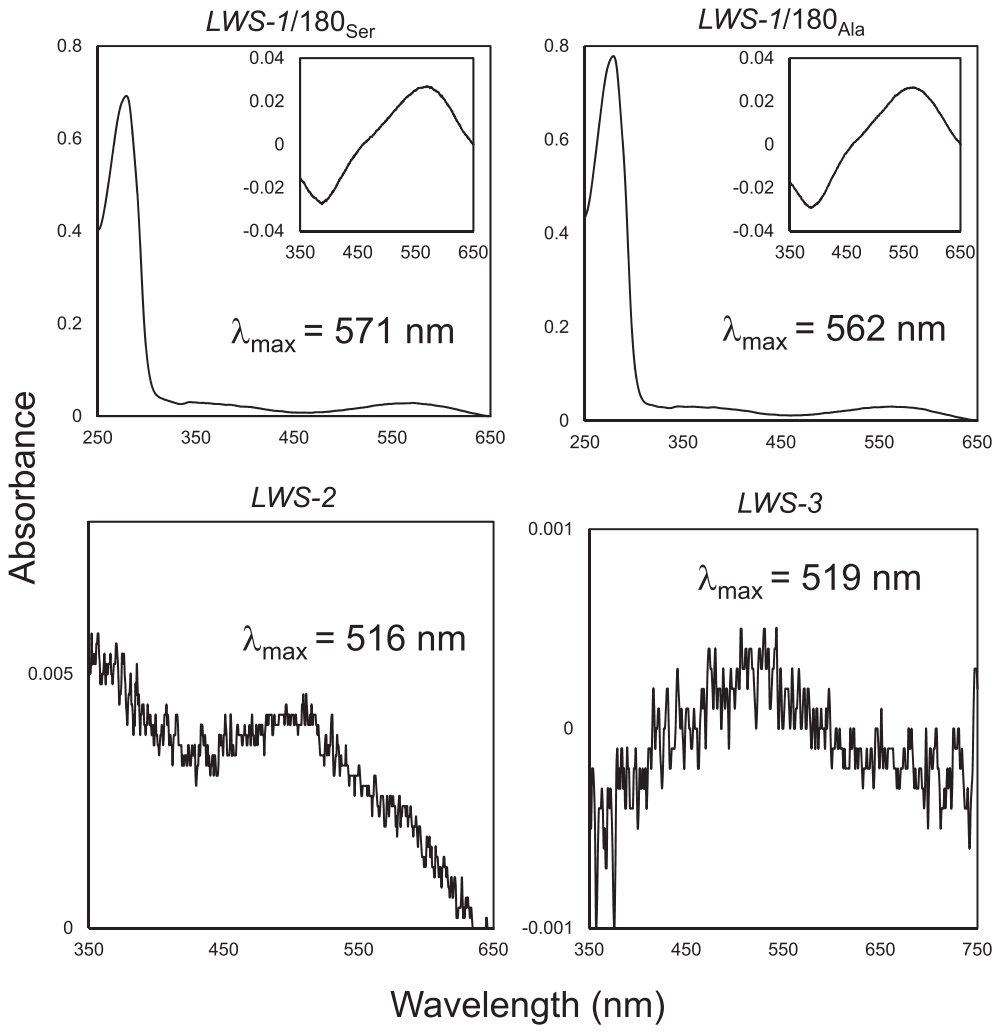

Fig. 1. Absorption spectra of *LWS* opsins reconstituted *in vitro*. The dark spectra and their λ_{max} values are shown for *LWS-1*/180_{Ser} and *LWS-1*/180_{Ala} with difference spectra in insets. The difference spectra and their λ_{max} values are shown for *LWS-2* and *LWS-3*.

Given the novel finding of guppy visual opsin λ_{max} values presented in this study, and of the recent finding of 180_{Ser} as an allele of *LWS-1* (Tezuka et al., 2014) and of varying allelic frequencies of *LWS-1*/180_{Ser} and *LWS-1*/180_{Ala} among localities (Sandkam, Young, & Breden, 2015; Sandkam, Young, Breden, Bourne, & Breden, 2015; Tezuka et al., 2014), our understanding of association of opsins with cone types in guppy can be greatly promoted (the fifth column of Table 1). Archer and Lythgoe (1990) collected guppies from rivers in Trinidad including Aripo and reported that yellow cone cells have λ_{max} at 572 nm by the MSP method. The *LWS-1*/180_{Ser} allele is found common in northern Trinidad including the Aripo river (Tezuka et al., 2014). On the other hand, the Cumana guppy is reported to have yellow cones with λ_{max} at 560 nm by the MSP method and to have the *LWS-1*/180_{Ala} allele (Watson et al., 2011). The present study suggests that the two alleles of *LWS-1* (having λ_{max} at 571 and 562 nm, respectively) explain the difference of λ_{max} of yellow cones between the two MSP studies.

Our unexpected finding of the short λ_{max} (519 nm) for the guppy *LWS-3* could explain why the green-yellow cone is present in the guppy but is absent in the green swordtail (Watson et al., 2010), if we postulate that co-expression of *LWS-1* and *LWS-3* occurs in a subset of cones in closely related *Xiphophorus* and *Poecilia* fishes. This satisfies the Archer and Lythgoe (1990) co-expression model for the green-yellow cones. The difference of green-yellow cones in λ_{max} between Trinidad (centered at 548 nm) and Cumana (541 nm) guppies could also be explained by co-expression model because of their difference of the yellow cones.

The difference of the present co-expression model from the original Archer and Lythgoe model (1990) is that the co-expressed opsin with the yellow cone opsin (*LWS-1*) is not the green cone opsin. The green cone is present in both the green swordtail (Watson et al., 2010) and the guppy and cannot be compatible with *LWS-3* because of the long-wave absorption predicted for the green swordtail. Although λ_{max} values of the *LWS-2* and *RH2-1* photopigments of the green swordtail need to be confirmed, in the guppy the two opsins have similarly short λ_{max} values (~516 nm) and differ in major expression area in the retina (*RH2-1* in ventral retina and *LWS-2* in central and dorsal retina: Rennison et al. (2011)). Thus, the green cones can be assigned to either *RH2-1* or *LWS-2* in different part of the retina. This assignment precludes the necessity of postulating co-expression of different classes of opsins in the green-yellow cones: an RH2 opsin (*RH2-1*) and an M/LWS opsin (*LWS-1*). However, the problem still remains: the λ_{max} values of the two opsins are shorter than the MSP values of the green cones in both Trinidad and Cumana guppies (the fourth and fifth columns of Table 1). Further survey of allelic polymorphism of *LWS-2* and *RH2-1* and improvement of their *in vitro* reconstruction could finally solve this problem.

We still have no clear association of *LWS-4* and *SWS2-A* with cell types (the fourth and fifth columns of Table 1). *LWS-4* is expressed only in the peripheral dorsal–temporal retina (Rennison et al.,

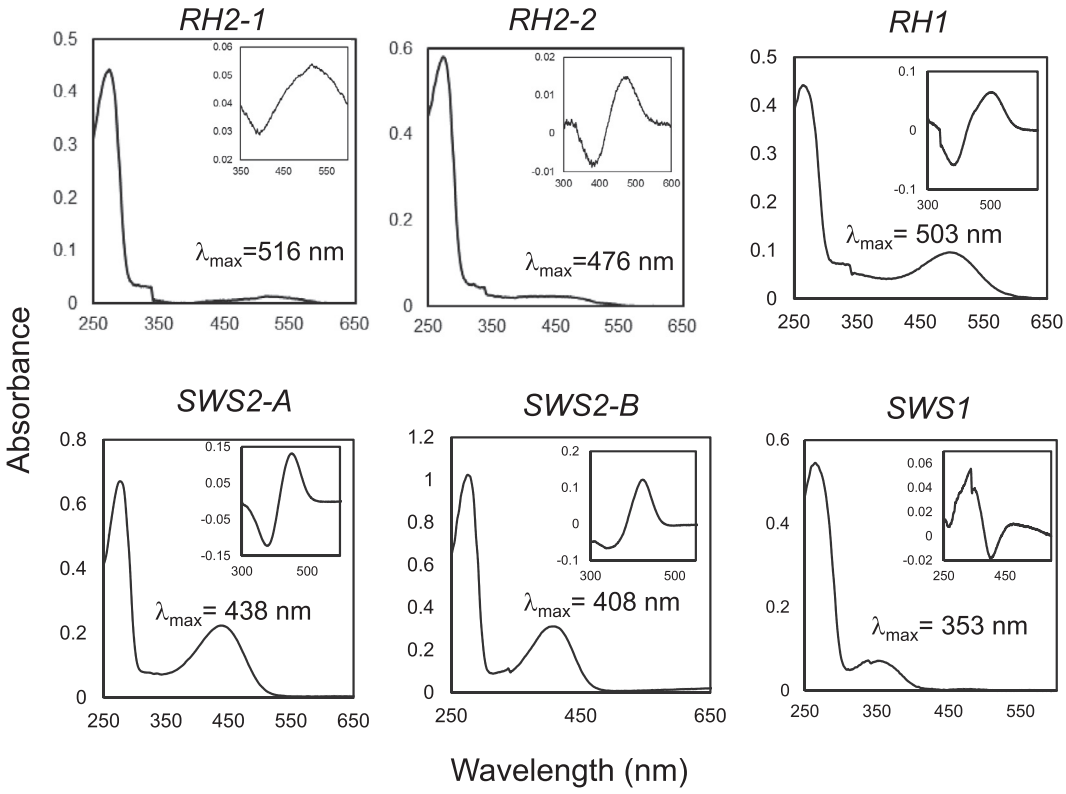

Fig. 2. Absorption spectra of *RH2*, *RH1*, *SWS2* and *SWS1* opsins reconstituted *in vitro*. The dark spectra and their λ_{max} values are shown with difference spectra in insets. The discontinuity of absorbance curve at around 350 nm appeared in the charts except of *SWS2-A*. This is due to an electronic noise when light sources changed between ultraviolet and visible light.

2011) and its expression level is low (Laver & Taylor, 2011; Sandkam, Young, & Breden, 2015). Considering this expression pattern, the role of *LWS-4* in vision may be negligible, if any. *SWS2-A* is expressed uniformly throughout the retina but its expression is much lower than *SWS2-B* (Laver & Taylor, 2011; Rennison et al., 2011; Sandkam, Young, & Breden, 2015). *SWS2-B* is also expressed uniformly throughout the retina (Rennison et al., 2011). *SWS2-A* (λ_{max} at 438 nm) may be co-expressed with *SWS2-B* (408 nm); Archer and Lythgoe (1990) found that the measured absorption curve of the violet cones is slightly long-wave shifted relative to the best fitting template. Thus, we need further endeavor to decipher visual system of the guppy.

Despite a few unresolved associations, we emphasize the importance of direct measurement of opsin absorption spectra to provide a solid basis for understanding the evolution of the visual system. Comparison of opsin absorption spectra between populations (e.g. *LWS-1* between Trinidad and Cumana guppies) and between species (e.g. *LWS-3* between guppy and swordtail) is also key to understanding color vision variation among them. With results we generate in this study, M-L cone spectral variation of guppies described by Archer and Lythgoe (1990) can be interpreted with a new view: the relative expression level among *LWS-1*, *LWS-2* and *LWS-3* may differ substantially among individuals, resulting in the broad λ_{max} range of the green-yellow cones and the various ratio of the yellow, green-yellow and green cones in the guppy retina. The expression plasticity of opsins in evolution and in ontogeny is a focus of recent studies on guppies under varying predation and lighting conditions (Sakai, Ohtsuki, Kasagi, Kawamura, & Kawata, 2016; Sandkam, Young, & Breden, 2015). Deciphering genetic mechanism behind the plasticity of opsin expression will further promote our understanding of the color vision, behavior and ecology of these tropical fishes.

Conflict of interest

The authors declare no conflict of interests.

Author contributions

1: Conception of the research, 2: Design of the experiments, 3: Collection of data, 4: Analysis of data, 5: Interpretation of data, 6: Drafting the manuscript, 7: Revising the manuscript critically for important intellectual content.

S. Kawamura: 1,2,4,5,6,7; S. Kasagi: 2,3,4,5,6; D.K.: 3,4; A.T.: 2,3; A.S.: 3,4; A.T.: 3; H.I.: 3,5, M.K.: 1,2,3.

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